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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: July 17, 1975

1. Principal Investigator (give title and degrees): Harry L. Ioachim, M.D.
-Clinical Professor of Pathology, College of Physicians and Surgeons of Columbia University
-Attending Pathologist, Lenox Hill Hospital

2. Institution & address:
Lenox Hill Hospital
100 East 77 Street
New York, New York 10021

3. Department(s) where research will be done or collaboration provided:

Department of Pathology

4. Short title of study:

The Immune Response at the Tumor Site in Lung Carcinoma

5. Proposed starting date: January 1, 1976

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

see appendix #1

Appendix #1

Based on the hypothesis that the tumor site contains the highest concentration of tumor-associated antigens and includes the specifically-sensitized lymphoid cells as well as the tumor-directed antibodies that they produce, our research aims will be:

1. The characterization of reactive histologic patterns associated with various types of lung carcinomas.

2. The functional identification of lymphocytes and plasma cells of the stromal reaction of lung carcinomas (i.e. T-cells, B-cells, IgG-, IgM-, IgA-secreting cells, etc.).

3. The isolation of specific antibodies from solid lung carcinomas and their pleural effusions.

4. The extraction, purification and characterization of antigens associated with lung carcinomas.

8. Brief statement of working hypothesis:

see appendix #2

9. Details of experimental design and procedures (append extra pages as necessary)

see appendix #3

Appendix #2

Previous work in this (1-4) and other (5-10) laboratories indicates the existence of tumor-associated antigens in human cancer. The present search for antigens specific for different types of tumors is strongly stimulated by the potential application of such antigens in the early diagnosis of cancer. Similarly, the identification of specific antibodies is of great practical importance for their use in immunodiagnosis and immunotherapy.

At the present time, the investigation of specific antigens in cancer patients is based primarily on skin tests (11-13) and on assays of lymphocyte reactivity to tumor extracts and tumor cells (14-16). A sustained effort is also being made to detect circulating antigens and antibodies in sera of cancer patients and several tumor-associated antigens have been described (1-2, 5-10, 17-18). However, to date, most of these efforts have not yet produced the expected results, possibly because tumor antigens in the circulating blood are diluted to infinitesimal amounts or locked in antigen-antibody complexes. Similarly, the lymphoid cells specifically primed to react to a particular tumor are highly diluted in the pool of circulating lymphocytes.

Based on our earlier studies (4, 19), it appeared to us that the tumor site would be a more logical place to explore the presence of tumor-reactive populations of lymphoid cells as well as to attempt the detection of tumor-associated antigens and antibodies.

Thus, the working hypothesis of this project is that the tumor itself with its reactive stroma is the site where specific antigens and antibodies could be expected to be present in highest concentration

Appendix #3

1. The characterization of reactive histologic patterns associated with various types of lung carcinomas.

Recent work from this laboratory (19) indicates that the stromal cellular reaction of lung carcinomas correlates with the histologic types and the degrees of differentiation of the tumors. Distinctive patterns of stromal cellular reaction, characteristic for different histologic types of lung carcinoma, were thus recognized. The amount of cellular infiltration was highest in squamous cell carcinomas and lowest or non-existent in oat cell carcinomas. Within the various histologic categories the well differentiated tumors appeared to be accompanied by more reactive cells than the poorly differentiated and there was no relation between tumor necrosis and cellular infiltration. The plasma cells were distinctly associated with squamous cell carcinomas and their amount in the stroma was proportionate to the degree of differentiation and the presence of keratin produced by the tumors.

Based on these observations we propose to undertake a study of clinical-pathologic correlations in which the patterns of stromal cellular reaction will be investigated and corroborated with the tumor type and grade as well as with the clinical history of the patients. To characterize the tumors we will consider their histologic diagnosis, degree of differentiation, amount of necrosis and capacity for invasion (vessels, lymph nodes, and pulmonary tissues). These features will be compared with the cellular reaction at the tumor site. The latter will be estimated according to several

parameters: amount, composition and distribution of both cellular infiltrates and of fibrous components. Details of these criteria are given in a recent study on the stroma reaction of lung carcinomas (19).

These data will be correlated with the clinical information available in the history of the patients such as age, sex, time from first tumor symptoms, clinical stage, treatment and survival. The total count of lymphocytes in the peripheral blood and the number of T-lymphocytes will be also considered since it was shown in a recent study from this laboratory (22) that the levels of T-lymphocytes were significantly decreased in patients with breast cancer.

2. The functional identification of lymphocytes and plasma cells of the stromal reaction of lung carcinomas.

The presence of large numbers of plasma cells and lymphocytes in the stroma of lung carcinomas and their selective association with different types of tumors (19) suggests that specific antibodies might be released by these cells.

In our experience, when cryostat sections of lung carcinomas were stained with anti human immunoglobulins in direct immunofluorescence, the cellular infiltrates, plasma cells and lymphocytes appeared positive for various immunoglobulins, IgG, IgM and IgA. The eluates which we recovered subsequently from such tumors reacted positively in indirect immunofluorescence with tissue-cultured lung adeno and squamous carcinoma cells but not with cell sus-

pensions of normal lung or tissue-cultured cells of various non pulmonary cancers.

In the present study we plan to attempt the identification of various types of lymphoid cells as well as the investigation of their secretory activity. Several techniques that have been recently set up in our laboratory will be used for this purpose. To determine if the reactive lymphocytes in the tumor stroma belong to the T or B classes of cells, lung carcinoma tissues will be minced and the resulting cell suspension will be separated into lymphoid cells and tumor cells with the aid of a modified Ficoll-Hypaque technique (30). The lymphoid cells thus separated will then be reacted with sheep erythrocytes (SE) to determine the numbers of T cells, as previously described (22) and with erythrocytes coated with antibody and complement (EAC) to determine the numbers of B cells (31).

To observe the location within the tumor tissues of T and B lymphoid cells we will also use the SE and EAC techniques on cryostat sections according to the methods described by Jaffe et al (31) which have already been used in our laboratory. The classes of immunoglobulins secreted by the lymphocytes and plasma cells of the stromal reaction will be determined by specific staining with fluorescein-labelled anti human IgG, IgM and IgA antisera and subsequent examination under ultra violet light (indirect immunofluorescence).

To permit the ultramicroscopical visualization of immunoglobulins on the surface of lymphoid cells we will employ a similar technique, previously used in our laboratory (32) by which ultra-thin sections of lung carcinomas will be reacted with peroxidase-labelled anti human immunoglobulins and examined under the electron microscope.

3. Isolation of specific antibodies from solid lung carcinomas and their pleural effusions.

Results of work done in our laboratory over the last year strongly suggest the existence of tumor specific autologous antibodies in patients with carcinoma of the lung.

Antibodies eluted from solid lung tumors have reacted positively in the indirect immunofluorescence test with both autologous and allogeneic lung tumor cells while failing to react with other normal or malignant cells. The recovery of such autologous anti-tumor antibodies from pleural effusions have been successfully attempted with quantitatively superior results. It appears that certain malignant effusions in lung carcinoma may represent a rich source of tumor specific antibody although its apparent activity may be masked by immune complex formation with shed soluble antigens present in the fluid.

Antibodies derived from lung carcinoma pleural effusions have demonstrated at least partial cross reactivity between cells derived from both squamous cell carcinoma and adenocarcinoma of the lung, while failing to react with cells derived from oat cell carcinoma of the lung. Conversely, such antibody activity has been demonstrated for epidermoid and adenocarcinoma but not for oat cell carcinomas. The antibodies recovered from malignant pleural effusions have been tested for reactivity with known tumor associated antigens including CEA by the radioimmunoassay and found to possess no such reactivity.

The present proposal projects the expansion of our findings according to the following plan.

Recovery of higher yields of tumor specific antibody from malignant effusions.

Although substantial yields of tumor reactive antibody (up to a titer of 1:64 in I.F.) have been prepared in this laboratory by acid disassociation and salt precipitation, results of preliminary experiments in this and parallel systems indicate that much higher yields of antibodies might be recovered. Recovery of significantly greater titers of antibody activity may allow the use of more informative but less sensitive analytic techniques such as double immunodiffusion. Such improved antibody recoveries can be obtained in two ways according to our experience:

a) Batch processing by ion exchange resins to prepare pure γ- globulin fraction (including antigen antibody complexes).

When large volumes of malignant effusions are received they are immediately dialysed to lower their ionic strength. They are then mixed with an amount of preswollen ion exchange resin capable of binding 2x the total protein content of the fluid and agitated for one hour. The ionic strength and pH of the slurry are adjusted to a range where only Ig G and Ig G complexes will elute from the gel and the liquid phase recovered by filtration. The resultant clear fluid contains only Ig G and is easily concentrated by ultrafiltration (20,21).

b) Separation of disassociated immune complexes on cation exchange resins at low pH.

Concentrated Ig G, Ig G-antigen complex fractions are subjected to low pH to disrupt immune complexes and absorbed to cation exchange resin columns at low pH. The columns are then eluted at a pH slightly below the isoelectric point of Ig G. This elution removes all serum protein from the column including antigen liberated from immune complexes. The bound Ig G is then removed by adjusting the pH to above the isoelectric point of Ig G while minimizing the loss of antibody activity due to acid hydrolysis.

In all these procedures the quantity and purity of immunoglobulin fractions are ascertained by radial immunodiffusion and immunoelectrophoresis in 1.5% agarose (19). The specific reactivity of recovered antibodies is determined by indirect immunofluorescence tests as previously described (23,24) using both cell suspensions obtained directly from tumors and cells from tissue culture as antigen substrates.

provide a simple method for evaluation of antigen recovery during purification.

Purification and characterization of antigens will be attempted by fractionation of crude tumor extracts using ion exchange chromatography combined with molecular sieve chromatography.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

see appendix #4

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

see appendix #5

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

see appendix #6

Appendix #4

Facilities Available

The Cancer Research Unit located at the 12th floor of the Lenox Hill Hospital includes 3 spacious, air-conditioned laboratories, 1 tissue culture room, 1 equipment room, and the electron microscopy laboratory.

The tissue culture room is fully equipped with ultra violet sterilizing lamps. sterile-work hoods, 4 CO_2-gassed incubators and 2 inverted microscopes for tissue cultures.

The immunology equipment includes:

1 Beckman ultracentrifuge

1 Sorvall centrifuge (Super Speed)

1 MSE Sonifier

2 Leitz Microscopes for Immunoflourescence with photo equipment

1 Revco deep freeze

1 Column chromatography set up, including a variety of experimental and preparative columns, a continuous flow U.V. monitor and recorder and a 200 tube automated fraction collector

1 Lyophilizer

1 Agarose immunoelectrophoresis equipment

1 Amicon 8 cell experimental ultrafiltration device

In addition our laboratory has in its inventory:

1 Liquid nitrogen

4 large freezers and refrigerators

1 Cryostat microtome

1 Three channel 100 sample automated Beta Counter

1 Three channel 200 sample automated Gamma Counter

The electron microscopy laboratory includes one 101 Siemens and RCA electron microscopes, 2 ultramicrotomes and a photography lab.

There are also animal facilities perfectly adequate for several hundred laboratory animals, including mice, rats, guinea pigs and rabbits. A separate mating room has allowed us to raise our own colonies of inbred animals, decreasing substantially the need for purchase.

A full-time animal caretaker and a full-time research assistant preparing and sterilizing the glassware are being entirely salaried by Lenox Hill Hospital.

Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Harry L. Ioachim	Clinical Professor	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED	REDACTED	REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
1. Lyceum "Cultura"-Bucharest	Baccalaureat	1943
2. Faculty of Medicine- Univ. of Bucharest	M.D.	1951
3. Dept. of Pathology- Faculty of Medicine Univ. of Bucharest	Instructor; Asst. Prof.	1951-
4. First Surgical Clinical- Faculty of Medicine. Univ. of Bucharest	Head of Path.	1951-
5. Institut du Cancer-Villejuif-Paris	Chargé de Recherches	1961-
6. Dept. of Path.-College of Phys.& Surg.-Columbia Univ	— Asst. Prof.	1962
	— Assoc. Clin Prof.	1968
	— Clin. Professor	1973-
7. Dept of Path. Lenox Hill Hospital	Attending Pathol.	1968-
8. New York State Board Licence	M.D.	1967
9. Board of Pathology	Pathologist	1967

HONORS

Career Scientist Award of the Health Research Council of N.Y.-1956
Awardee of National Cancer Institute-Grants in aid- 1967.1970.1973
Awardee of New York Cancer Research Institute,-Grant in aid-1970
Permanent Editor of "Pathobiology Annuals"-Appleton-Century-Crofts. publishers, first five volumes published: 1971-1975

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Tumor Immunology. Leukemia, Oncogenic Viruses	Principal Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Twenty five years of practice in pathology
Twenty years of activity in Experimental and Clinical Oncology
Ninety eight publications in Pathology and Oncology

Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Brent H. Dorsett	Research Associate	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED		REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
Brooklyn College City University of New York	B.S. Biochemistry	1968

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Human Tumor Immunology	Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Ten years of experience in research immunology
15 publications in fields of virology and immunology (see attached lists)
Research Assistant, Department of Biophysics, Mellon Institute,
Pittsburgh, Pa. 1964-1965
Research Assistant, The Rockefeller University, New York City 1965-1967
Research Assistant and Lecturer, Virus Chemistry Research Laboratory
City University of New York 1967-1970
Senior Research Assistant, Virology Division Sloan Kettering Institute
for Cancer Research, New York 1970
Research Associate, Cancer Research Unit, Department of Pathology
Lenox Hill Hospital 1970 to present

Appendix #5

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE
Edward P. Paluch	Research Associate	REDACTED

PLACE OF BIRTH	NATIONALITY	SEX
REDACTED	REDACTED	REDACTED

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR
University College New York University	B.A. Biology	1974
Columbia University	Ph.D. Candidate	

MAJOR RESEARCH INTEREST	ROLE IN PROPOSED PROJECT
Human Tumor Immunology	Investigator

RESEARCH AND PROFESSIONAL EXPERIENCE

Three years of experience in research immunology
4 publications in the field of immunology (see attached lists)
Research Assistant, Department of Pathology, Lenox Hill Hospital
100 East 77 Street, New York, N.Y. 1970-1971
Research Associate, Cancer Research Unit, Department of Pathology
Lenox Hill Hospital. 1972 to present

Appendix #6

PUBLICATIONS FROM THIS LABORATORY PERTAINING TO THE TOPICS OF THIS PROPOSAL

1. Dorsett B.H., Ioachim H.L. - Common antigenic component in ovarian carcinomas: demonstration by double diffusion and immunofluorescence techniques. Immunol. Communications. 2:173-184, 1973.
2. Ioachim H.L., Dorsett B.H., Sabbath M., Andersson B. and Barber H.R.K. - Antigenic and morphologic properties of ovarian carcinoma. Gynecol. Oncology. 1:130-142, 1973.
3. Ioachim H.L., Sabbath M., Andersson M., and Barber H.R.K. - Tissue cultures of ovarian carcinomas. Lab. Invest. 31:381-390, 1974.
4. Dorsett B.H., Ioachim H.L., Stolbach L. and Barber H.R.K. - Isolation of tumor specific antibodies from effusions of ovarian carcinomas - submitted for publication.
5. Ioachim H.L., Dorsett B.H., Paluch E. and Barber H.R.K. - Electron microscopy, tissue cultures and immunology of ovarian carcinoma. Symposium on Ovarian Carcinoma, Reston, Virginia November 1974 - National Cancer Institute, - Monograph, in print.
6. Dorsett B.H., Paluch E. and Ioachim H.L. - Circulating antibodies reacting with epidermal cells in patients with squamous cell carcinoma of the lung - Feder. Proc. 33:597, 1974.
7. Ioachim H.L., Dorsett B.H., and Paluch E. - Cellular and humoral immune reactions to squamous cell carcinoma of the lung. XIth International Cancer Congress, Florence, Italy. 2:104, 1974.

Appendix #6 (cont.)

8. Ioachim H.L., Dorsett B.H. and Sabbath M. - The immune response at the tumor site in lung carcinoma: evaluation of cellular reaction and recovery of tumor specific antibodies - submitted for publication.

9. Ioachim H.L., Keller S.E., Sabbath M. and Dorsett B.H. - Antigenic expression as a determining factor of tumor growth in Gross Virus Lymphoma - Progr. Exp. Tumor Res. 19:284-296, 1974.

10. Ioachim H.L., Keller S.E., Dorsett B.H. and Pearse A. - Induction of partial immunologic tolerance in rats and progressive loss of cellular antigenicity in Gross Virus Lymphoma - J. Exp. Med. 139:1382-1394, 1974.

11. Ioachim H.L., Keller S.E., and Dorsett B.H. - Transplantability, Immunological Unresponsiveness and Loss of Cellular Antigenicity in Gross Virus Lymphoma - in Symposium on Leukemogenesis - 3:301-310, Tokyo 1975.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
Harry L. Ioachim, M.D.	-Principal Investigator	30%	none
Sheldon C. Sommers, M.D.	-Consultant	10%	none
Brent H. Dorsett, B.S.	-Investigator	80%	
Edward Paluch, B.S.	-Investigator	30%	REDACTED
Technical			
Caren Colbjornson	-Technician	70%	
Tove Bamberger	-Secretary	40%	REDACTED

Sub-Total for A REDACTED

B. Consumable supplies (by major categories)

Reagents (immunology)	1,500
Glassware, tissue culture media	1,500
Electron Microscopy (supplies and services)	1,500

Sub-Total for B 4,500

C. Other expenses (itemize)

Publication costs	500
Travel	500
Miscellaneous	500

Sub-Total for C 1,500

Running Total of A + B + C

D. Permanent equipment (itemize)

None

Sub-Total for D 0

E. Indirect costs (15% of A+B+C) E

Total request $30,000

15. Estimated future requirements:

		Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	R		6,600	-	-		33,000
Year 3	R		7,260	-	-		36,300

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies of Induction and Immunity of Experimental Lymphomas	National Cancer Institute CA 16997	40,000	1974-1977

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Detection of Antigens and Antibodies in Cancers of the Ovary	National Cancer Institute (planned)		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Harry L. Joachim

Signature [signature] Date 7/17/75

Telephone 212 (Area Code) 794-4420 (Number) (Extension)

Checks payable to

Lenox Hill Hospital

Mailing address for checks

100 East 77 Street

New York, New York 10021

Responsible officer of institution

Typed Name Louis F. Parker

Title Vice President

Signature [signature] Date 7/17/75

Telephone 212 (Area Code) 794-5141 (Number) (Extension)

REFERENCES

1. Dorsett B.H. and Ioachim H.L. - Common antigenic component in ovarian carcinoma: demonstrations by double diffusion and immuno-fluorescence techniques - Immunological Communications 2:173-84, 1973.

2. Dorsett B.H., Paluch E. and Ioachim H.L. - Circulating antibodies reacting with epidermal cells in patients with squamous cell carcinoma of the lung - Feder. Proc. 33:597, 1974.

3. Ioachim H.L., Dorsett B.H. and Paluch E. - Cellular and humoral immune reactions to squamous cell carcinomas of the lung - XIth International Cancer Congress, Florence, Italy 2:104, 1974.

4. Ioachim H.L., Dorsett B., Paluch E., Barber H.R.K. - Electron microscopy, tissue cultures and immunology of ovarian carcinoma - Symposium on ovarian carcinoma - National Cancer Institute, Reston, Virginia November 1974.

5. Gold P., Freedman S.O. - Demonstration of tumor specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. - J. Exp. Med. 121, 439-462, 1965.

6. Klein G., Clifford P., Klein E. and Stjernsward J. - Search for tumour-specific immune reactions in Burkitt lymphoma patients by the membrane immunofluorescence reaction - Proc. Nat. Acad. Sci. USA 55:1628-1635, 1966.

7. Hellstrom I., Hellstrom K.E., Pierce G.E. and Yang J.P.S. - Cellular and humoral immunity to different types of human neoplasms Nature (London) 220, 1352-1354, 1968.

8. Morton D.L., Malmgren R.A., Holmes E.C. and Ketcham A.S. - Demonstration of antibodies against human malignant melanomas by immunofluorescence - Surgery 64:233-240, 1968.

9. Hellstrom I., Hellstrom K.E., Evans C.A., Heppner G.H., Pierce G.E., Yang J.P.S. - Demonstration of cell-bound and humoral immunity against neuroblastoma cells. - Proc. Nat. Acad. Sci. USA 62:362-369, 1969.

10. Bubenik J., Perlmann P., Helmstein K., Moberger G. - Cellular and humoral immune responses to human urinary bladder carcinomas. Int. J. Cancer 5:310-319, 1970.

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12. Stewart T.H.M. - The presence of delayed hypersensitivity reactions in patients toward cellular extracts of their malignant tumors. - Cancer 23:1368-1369, 1969.

13. Eltringham J.R., Kaplan H.S. - Impaired delayed-hypersensitivity responses in 154 patients with untreated Hodgkin's disease - Natl. Cancer Inst. Monogr. 36:107-115, 1973.

14. Hersh E.M., Irwin W.S. - Blastogenic responses of lymphocytes from patients with untreated and treated lymphomas - Lymphology - 2:150-160, 1969.

15. Whitaker M.G., Clark C.G. - Depressed lymphocyte function in carcinoma of the breast - Br. J. Surg. 58:717-720, 1971.

16. Catalona W.J., Sample W.F., Oretien P.B. - Lymphocyte reactivity in cancer patients: correlations with tumor histology and clinical stage - Cancer 31:65-71, 1973.

17. Bhattacharya M., Barlow J.J. - Immunologic studies of human serous cystadenocarcinoma of ovary - Cancer 31:588-595, 1973.

18. Hirshaut Y., Pei D.T., Marcove R.C., Mukherju B., Spielvogel A.R. and Essner E. - Seroepodemiology of Human Sarcoma Antigen (S_1) - N. Engl. J. Med. 291:1103-1107, 1974.

19. Ioachim H.L., Dorsett B.H. and Sabbath M. - The immune response at the tumor site in lung carcinoma: evaluation of cellular reaction and recovery of tumor specific antibodies - submitted for publication.

20. Baumstark J.S., Laffin R.J., Bardawil W.A. - A preparative method for the preparation of 7S gammaglobulin from human serum - Arch. Biochem. Biophys. 108:514-522, 1964.

21. Perper R.J., Okimoto J.T., Cochrum K.C. - A rapid method for purification of large quantities of antilymphocytic serum - Proc. Soc. Exptl. Biol. Med. 125:575-580, 1967.

22. Keller S.E., Ioachim H.L., Pearse T., and Silletti D.M. - Decreased T-lymphocyte levels in patients with breast cancer - Amer. J. Clin. Pathol. (in press).

23. Ioachim H.L., Keller S.E., Sabbath M. and Dorsett B.H. - Antigenic expression as a determining factor of tumor growth in Gross Virus Lymphoma - Progr. Exp. Tumor Res. 19:284-296, 1974.

24. Ioachim H.L., Keller S.E., Dorsett B.H. and Pearse A. - Induction of partial immunologic tolerance in rats and progressive loss of cellular antigenicity in Gross Virus Lymphoma - J. Exp. Med. 139:1382-1394, 1974.

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29. Ohno Y., Stahmann M.A. - A new detection method for penicillin antibodies and hypersensitivity in humans using penicillin coupled to polyacrylamide particles. Immunochemistry 9:1078-1093, 1972.

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30. Fortino M., Merson F.J., Allen F.H. - Micromethod for separation of lymphocytes from peripheral blood. Ann. Clin. Lab. Sci. 1:131-133, 1971.

31. Jaffe E.S., Shevach E.M., Frank M.M., Berard C.W., and Green I. - Nodular Lymphoma - evidence for origin from follicular B lymphocytes. N. Engl. J. Med. 290:814-819, 1974.

32. Schmidt E.C., Dorsett B.H., Paluch E., and Ioachim H.L. - Membrane immunoglobulins of human lymphoma cells studied by immunoelectron microscopy. J. Cell Biol. 63:301, 1974.

THE IMMUNE RESPONSE AT THE TUMOR SITE IN LUNG CARCINOMA: EVALUATION OF CELLULAR REACTION AND RECOVERY OF TUMOR SPECIFIC ANTIBODIES.

Submitted for publication
July 1975.

Harry L. Ioachim, M.D., Brent H. Dorsett, B.S. and
Marlene Sabbath, M.S.

Departments of Pathology of Lenox Hill Hospital and
College of Physicians and Surgeons of Columbia University,
New York.

Title for table of contents:

Cellular reaction and tumor specific antibodies in lung carcinoma.

Running title:

Immune response in lung carcinoma.

Acknowledgements

Grateful appreciation to Dr. Sheldon C. Sommers, Professor of Pathology for critical review of this manuscript and to Mrs. Rose Mary Spitalieri and Tove Bamberger for photographic and secretarial help.

Financial support was provided by Grant No. 945R1 from the Council for Tobacco Research.

Address reprint requests to:

Harry L. Ioachim, M.D., Lenox Hill Hospital, 100 East 77th Street, New York, N.Y. 10021

Abstract

The local immune response to lung cancer was investigated by histologic and immunologic means. Distinctive patterns of stromal cellular reaction, characteristic for different histologic types of lung carcinoma, were recognized. The amount of cellular infiltration was higest in squamous cell carcinomas and lowest or non-existent in oat cell carcinomas. Within the various histologic categories the well differentiated tumors appeared to be accompanied by more reactive cells than the poorly differentiated and there was no relation between tumor necrosis and cellular infiltration. The plasma cells were distinctly associated with squamous cell carcinomas and their number in the stroma was proportionate to the degree of differentiation and the presence of keratin produced by the tumors.

Eluates with a high content of immunoglobulins were recovered from pleural effusions and from solid lung carcinomas by dissociation of antigen-antibody complexes. These preparations reacted positively in indirect immuno-fluorescence tests with tissue cultures and with fresh suspensions of lung carcinoma cells but not with tissue culture cells of most non pulmonary tumors or of normal adult and fetal lung. Similarly prepared fractions of non carcinomatous pleural effusions did not react with lung cancer cells.

According to modern concepts of tumor immunology, cancer cells may express altered antigenic properties acquired as a result of neoplastic transformation. The existence of tumor-associated antigens has been clearly demonstrated in both experimental (1-8) and human (9-23) tumors.

In cancer patients the search for specific tumor antigens is based primarily on skin tests (14-15,17,24-29) and on assays of lymphocyte reactivity to tumor extracts and tumor cells (17,29-36). A sustained effort is also being made to detect circulating antigens in sera of cancer patients and several tumor-associated antigens have been described (6,9-13,18-23,37-39). However, to date, most of these efforts have not yet produced the expected results, possibly because tumor antigens in the circulating blood are diluted to infinitesimal amounts or

locked in antigen-antibody complexes. Similarly, the lymphoid cells specifically primed to react to a particular tumor are highly diluted in the pool of circulating lymphocytes.

It appeared to us that the tumor site would be a logical place to explore the presence of tumor-reactive populations of lymphoid cells as well as to attempt the detection of tumor-associated antigens and antibodies.

To evaluate the morphology of the local cellular reaction to cancer of the lung we examined comparatively sections of 50 cases of different types and grades of lung carcinomas. The aim was not to establish clinical-pathologic correlations but to assess the presence of a cellular reaction and to estimate its intensity and its composition in regard to the various types and grades of lung carcinomas. To investigate the function of these cellular infiltrates we attempted the recovery, identification and quantitation of the immunoglobulins at the tumor site. Using solid tumors and pleural effusions, we obtained eluates with a high content of immunoglobulins showing tumor specificity. Considering that a large amount of antibodies are probably bound in antigen-antibody complexes we applied techniques designed to dissociate such complexes and to permit the recovery of active antibodies.

Materials and Methods

A. Pathology

1. Selection of Cases

Fifty recent consecutive cases of lung carcinomas from the files of our department, were studied. All were surgical specimens, lobectomies or pneumonectomies, that were routinely processed for microscopic examination. In each case an average of 10 slides comprising Bouin-fixed, trichrome-stained (phloxine-hematoxyline-safranin) sections were examined.

2. Evaluation of Tumor.

To characterize the tumor we considered its histologic diagnosis, degree of differentiation, amount of necrosis and capacity for invasion. The lung carcinomas examined were diagnosed as squamous, adeno, bronchiolo-alveolar, oat cell and undifferentiated carcinomas.

The degrees of differentiation were estimated on a scale from 1 to 4 as undifferentiated -1-, poorly -2-, moderately -3-, and well differentiated -4-. The amount of necrosis was similarly evaluated from 1 to 4, in relation to the total mass of tumor tissue in a microscopic field.

The capacity for tumor invasion was estimated in relation to the invasion of pulmonary tissues, blood vessels and hilar lymph nodes. Tumor invasion of local tissues appeared as rounded nodules -1-, elongated

cords -2-, monocellular files -3- and single tumor cell invasion -4- and were considered to indicate increasing degrees of aggresiveness.

3. Estimation of Cellular Reaction

Three parameters were considered for the estimation of the cellular reaction accompanying the tumor: its amount, its composition and its distribution. The amount of cellular infiltrate was estimated on a scale from 1 to 4 by comparing in each microscopic field the surfaces occupied by tumor tissues and by cellular infiltrates, respectively. The composition of cellular reactions was marked with a P (plasma cells), L (lymphocytes) or N (neutrophils) when any of these types of cells constituted over 75 percent of the infiltrates and P.+ L (plasma cells + lymphocytes) when each of the latter were represented in about equal amounts. The distribution of the cellular infiltrate was considered in relation to the tumor cords as well as to bronchi and blood vessels. The reaction was designated PERI when the cellular infiltrate encircled only the periphery of the tumor nodule, INTER when it surrounded each tumor cord and INTRA when reactive cells penetrated between and/or in tumor cells.

The presence, amount and distribution of fibro-collagen were also estimated on a scale from 1 to 4.

Morphologic evidence of tumor cell destruction by reactive cells was

separately recorded. Also noted were occasional cellular infiltrates that have accumulated around bronchioles or blood vessels.

B. Immunology

1. Recovery of Immunoglobulins from Solid Tumors.

Solid tumors were finely minced and a 20% homogenate in saline prepared by twenty strokes of a tight fitting Dounce homogenizer at 4°C. The homogenate was slowly adjusted to pH 3.0 by the dropwise addition of 0.1N HCl and maintained at that pH for 30 minutes at 4°C with constant stirring. Acid insoluble precipitate was removed by centrifugation at 1500 x g for 10 minutes and the immunoglobulins were precipitated by slow addition of one-third volume of saturated NaCl. Precipitated immunoglobulins were collected by centrifugation at 1500 x g for 15 minutes and redissolved in borate buffered saline pH 8.0.

2. Recovery of Immunoglobulins from Tumor Effusions

Fluids of pleural effusions were collected under sterile conditions and clarified by centrifugation at 40,000 x g for 30 minutes at 4°C. The supernatant was precipitated by the slow addition of one-half vol. of saturated ammonium sulfate (SAS) at room temperature, with continuous stirring. After fifteen minutes, the precipitate was collected by centrifugation for 10 minutes at 5,000 x g. The pellets consisted primarily of gamma and beta globulins, as assessed by immunoelectrophoresis; they were redissolved in borate buffered saline pH 8.0 and the SAS precipitation repeated 2 times. The final pellets were redissolved in glycine-HCl buffer,

pH 2.8 at 25°C. The pH was monitored and maintained at pH 2.8 for 15 min. with constant stirring to allow for dissociation of antigen-antibody complexes. The solution was briefly centrifuged at high speed to remove any acid insoluble material and the immunoglobulin fraction recovered by precipitation with 1/3 volume of saturated sodium chloride. The precipitated globulins were immediately recovered by centrifugation for 10 minutes at 5,000 x g at 4°C and redissolved in borate buffered saline. The purity of the globulin fraction thus obtained was assessed by immunoelectrophoresis and the quantities recovered were determined by radial immunodiffusion.

3. Immunoelectrophoresis.

Analysis of immunoglobulins was performed by electrophoresis of samples in 1% agarose in barbitol buffer ionic strength 0.075 pH 8.6 with a constant current of 30 mA for 35 minutes at 4°C. Goat anti human serum antisera (Hyland Laboratories) was added to preformed troughs and allowed to diffuse for 24 hours at room temperature.

4. Quantitation of Immunoglobulins

Antibody yields were assessed by performing radial immunodiffusion tests in high and low level Quantaplates (Kallsted Laboratories). Quantitative determination were performed for IgG, IgA, IgM and IgD on all antibody preparations.

5. Tissue Cultures

Pleural effusion fluids collected under sterile conditions were sedimented by a 10 minute centrifugation at 200 x g then resuspended in 1-2 x 10^6 cells per ml in Eagle's minimal essential medium (MEM) supplemented with 15% fetal bovine serum and 10% human cord serum plus 1% antibiotics. The cells were grown as monolayers in Petri dishes placed in a CO_2-gassed incubator. Solid tumors were finely minced and in some cases trypsinized with 0.25 per cent trypsin in Hank's fluid for 30 minutes at 37°C. They were then resuspended in Eagle's medium and seeded as above. All cultures were subcultured by trypsinization at periodic intervals according to growth, and examined under the phase contrast microscope and on Giemsa-stained coverslips placed in the tissue culture dishes as previously described (40).

6. Direct Immunofluorescence of Tissue Sections.

Small portions of solid tumors for direct immunofluorescence were covered with OTC embedding compound (Ames Laboratories)and quicly frozen at -80°C. Cryostat sections 3 microns thick were air dried and fixed in acetone for 1 minute. Sections were covered with appropriately diluted FITC-labeled goat antihuman globulin antiserum and incubated at 37°C for thirty minutes. Sections were then washed 3 times for 5 minutes in phosphate buffered saline (PBS), and cover-slipped with 10% PBS pH 7.4 in glycerol.

7. Indirect Immunofluorescence of Cell Suspensions and Tissue Cultures.

Slides for cytoplasmic indirect immunofluorescence assays were prepared from both solid tumors and in vitro cultured cells. Solid tumors were finely minced and the resultant single cell suspension washed twice in PBS at pH 7.2. These cells or cells directly from tissue culture were suspended at a concentration of 1×10^6 cells/ml in Eagle's MEM medium with 10% fetal bovine serum. A drop of cell suspension was placed directly on each well of 8 spot fluoroglide-sprayed slides and incubated 17 hours in a humid 5% CO_2 atmosphere at 37°C to allow cell attachment. The slides were then washed for 10 minutes in PBS and fixed in acetone for 5 minutes. Slides thus prepared were stored at -80°C until used. Indirect immunofluorescence assays were performed by placing appropriate dilutions of antibody on each spot of prepared slides and incubated in a humid chamber for 30 minutes at 37°C . The slides were then washed three times in PBS for 5 minutes and once in deionized water for one minute. After air drying, appropriately diluted F.I.T.C. labeled anti human IgG, IgA or IgM was applied to each spot. The slides were incubated as before and washed 3 times for 5 minutes in PBS, counterstained in 0.01 % Evans blue for 30 seconds and washed 5 minutes in deionized water. After drying the slides were cover slipped with 10% PBS pH 7.4 in glycerol and examined by incident fluorescent illumination.

Results

A. Estimation of Cellular Reaction to Lung Carcinoma

Fifty lung carcinomas were examined according to various parameters and an attempt was made to establish correlations between the characteristics of the tumors and those of their stromal cellular reaction. All histologic types of lung carcinoma were represented in proportions similar to those usually encountered, (squamous cell carcinoma 21, adeno carcinoma 14, alveolar carcinoma 2, oat cell carcinoma 5, undifferentiated carcinoma 6 and metastatic adeno carcinoma 2.

The amount of tumor necrosis was inversely proportional to the degree of tumor differentiation, lowest (necrosis = 1.0) in the well differentiated alveolar carcinomas (differentiation = 4.0) and highest (necrosis = 2.8) in oat cell carcinomas (differentiation = 1.0). The invasion of the surrounding tissues was highest in oat cell carcinomas (tissue invasion = 3.8) and lowest in metastatic adeno carcinoma (tissue invasion = 1.5). The ratio of lymph nodes involved by metastatic carcinoma did not directly correlate with the histologic features being also related to the location, size and duration of tumor.

The amounts of cellular infiltration in and around lung carcinomas clearly correlated with their histologic types and degrees of differentiation. When the estimates of cellular amounts were averaged it was noted that well differentiated tumors had significantly more cellular infiltrates than poorly or undifferentiated tumors. Squamous cell carcinomas, well differentiated, (differentiation = 3.5) had the largest amounts of cellular infiltrates (reaction amount = 3.4) (fig. 1) whereas oat cell carcinomas (differentiation = 1.0)

were almost entirely devoid of cellular infiltrates (reaction amount = 0.7) (fig.2). This contrast between the very large cellular infiltrates of squamous cell carcinomas that sometimes exceeded the amount of tumor tissue and their absence in oat cell carcinomas was striking and consistent.

The distribution of reactive cells was mostly at the periphery of tumor nodules (PERI) in the less differentiated tumors and in between (INTER) tumor cords and tumor cells in the squamous cell carcinomas. When the amount of cellular infiltration was high (reaction amount = 3 to 4) such as in squamous cell carcinomas, lymphocytes were often seen within tumor cells (INTER)(fig. 3-4).

Destruction of tumor cells, singly or in groups, surrounded by abundant agglomerates of lymphocytes and plasma cells was frequently noted in these tumors (fig.4). The appearance of single tumor cell death due to lymphocytic activity was entirely distinctive from the ischemic necrosis of tumor tissue that involved larger areas and was not accompanied by lymphoplasmacytic infiltration (fig.5). Lymphocytes within epithelial tumor cells, previously described under the name of emperipolesis (41) was often observed in areas of tumor destruction (fig.3-4). Another unusual appearance, tumor cells engulfed by tumor cells was occasionally recorded (fig.6). As in our earlier observations this was noticed almost exclusively in squamous cell carcinomas, particularly those poorly differentiated with marked anaplasia and giant tumor cells (41).

Of all parameters used, the composition of cellular infiltrates correlated best with the histologic type of lung carcinoma. Plasma cells were present in large amounts in the squamous cell carcinomas (fig. 7) while few or none were seen in other histologic types. The well differentiated squamous cell carcinomas that included the formation of keratin, exhibited the greatest numbers of plasma cells. Not unusually in such tumors, plasma cells were the only constituent of stromal cellular reactions, occasionally forming huge sheets of cells that on occasion exceeded the size of tumor cords (fig. 1, 7). It appeared that a direct correlation existed between the presence of keratin or keratin precursors in squamous cell carcinomas and the amount of plasma cells in the surrounding stroma. In the adeno carcinomas, plasma cells were present mostly in areas of squamous metaplasia. In lymph nodes with tumor metastases, numerous plasma cells were present in contact with the tumor, particularly when this was squamous cell carcinoma. On occasion, huge areas of lymph nodes invaded by metastatic squamous cell carcinoma were transformed in sheets of plasma cells.

Fibrosis was noted in almost all lung carcinomas however its degree and distribution were variable. In general, the amount of fibro-collagenous tissue around (PERI) and within (INTER) the tumor nodules was indirectly proportional to the amount of cellular infiltrates. Tumors with abundant cellular reaction showed little proliferation of collagen fibers (fig. 1, 7) whereas tumors with few or no cellular infiltrates contained broad, dense bands of collagen (fig. 2). Even within the same tumor there were areas of fibrosis with poor cellularity, in contrast to areas of abundant cellular reaction that did not include a sizable fibrous component. Metastatic carcinomas to the lung showed patterns of

cellular infiltrates similar in size and composition to those of primary carcinomas of same histologic type.

B. Isolation of tumor specific antibodies

Cryostat sections of lung carcinomas were reacted with FITC-labeled antihuman IgG, IgM and IgA antisera and examined under the fluorescence microscope. It was observed that the plasma cells and lymphocytes of the stromal reaction exhibited strong intracytoplasmic fluorescence to all three immunoglobulins although IgG-positive cells were predominant (fig. 8). To investigate the nature and specificity of such immunoglobulins, eluates of lung carcinomas as well as fluids of pleural effusions were processed.

When solid lung tumors were eluted with acid buffer and the immunoglobulins recovered by salt precipitaton, an average of 2mgm IgG per gram of tumor tissue was obtained. IgA and IgM in substantially lower amounts were also recovered. Indirect immunofluorescence tests performed with these eluates and monospecific conjugates showed apparently specific binding of IgG to lung carcinoma cells grown in tissue culture. Three of four eluates of solid lung carcinomas (table 2) adjusted to a concentration of 2 mgm/ml and tested at a 1:10 dilution, exhibited granular cytoplasmic fluorescence with tissue culture cells of a pulmonary adenocarcinoma (fig. 9). In addition, two of these preparations gave similar immunofluorescence patterns with cultures of squamous cell carcinomas of the skin and larynx. All four eluates were negative when tested against normal fetal and adult lung cell suspensions as well as against tissue culture cells of non pulmonary carcinomas (table 2).

Pleural effusions accompanying lung carcinomas were also assayed for the presence of tumor specific antibodies. Freshly tapped fluids assayed in indirect immunofluorescence against lung carcinoma cells, did not show any reactivity. However, when the antigen-antibody complexes were dissociated, the IgG fraction recovered by salt precipitation exhibited substantial tumor reactive antibody activity. In immunoelectrophoresis this acid-treated immunoglobulin fraction was found to consist almost entirely of IgG with very little contamination of other serum proteins (fig.10).

IgG fractions prepared from two lung carcinoma effusions were tested in the indirect immunofluorescence assay and found to produce tumor cell staining patterns similar to those obtained with acid eluates of solid lung tumors (fig.11). Both of these IgG fractions prepared from 500 ml of effusion fluids consisted of about 50 ml of IgG at 4.0 mg/ml and gave positive tumor cytoplasmic immunofluorescence at a dilution of 1:64. The fractions reacted positively with tissue culture cells of both squamous cell and adeno carcinoma of the lung and failed to react with cell suspensions of normal human tissues or of tissue culture cells of non pulmonary carcinomas (table 3). Antibody fractions similarly prepared from pleural effusions of patients with lymphosarcomas, ovarian and breast carcinomas, oat cell pulmonary carcinoma and renal failure failed to give positive immunofluorescence staining with lung carcinoma tissue culture cells. In addition to the intracytoplasmic fluorescence produced by the antibody preparations of cancer effusions, nucleolar staining was also observed in all carcinoma cells reacted with these antibody fractions. This may indicate the presence of tumor-associated nucleolar antigens detected by our immunoglobulin extracts. Similar nucleolar antigens reacting with autologous and homologous sera in indirect immunofluorescence were recently described in melanoma patients (42).

Discussion

The infiltration of cancertissue by reactive cells has long been noticed by pathologists who have regarded it as an indication of host resistance to tumor invasion. To express this idea, Russel (1908) coined the term "stroma reaction" (43) while Murphy (1921) advanced the view that "immunity to cancer, whether natural or induced, is attended by lymphoid stimulation" (44-45). Ewing (1940) similarly regarded the inflammatory reaction accompanying tumors as a defensive process (46) and a number of subsequent histologic studies attempted to demonstrate a positive correlation between stromal reaction and prognosis (47-54). However, counter arguments were furnished by equally prestigious authors (Woglom (1929) (55) Willis (1953) (56)) as well as by several histologic studies (57-59), to show that the case for the tumor-antagonizing activity of lymphocytes was not yet proved (60).

It is notable that the concept of stromal reaction, which preceded by half a century the modern theories of cellular immunity to cancer, is not used for practical purposes today. In fact, the stromal reaction of tumors is hardly ever mentioned in pathology reports and there are no accepted criteria for its evaluation. To assess the significance of stromal reaction, standardized data regarding a large spectrum of tumors are obviously needed. On the other hand, the modern studies of cancer immunity that use a diversified and complex methodology to investigate the function of lymphocytes and antibodies seem to ignore the cellular reaction at the tumor site and are almost exclusively concerned with the cell populations of the general circulation.

Considering that the host's response to a tumor must be maximal at the site of the tumor, we have attempted the investigation of the local cellular and humoral reactions. To estimate the cellular infiltration at the tumor site, we have used a set of histologic criteria designed to permit quantification

and comparison. These criteria are certainly imperfect due to the many variables inherent to the study of tissue sections. However, they can provide, if consistently applied, a fairly accurate picture of the stromal reaction of various tumors. Thus it was possible to recognize patterns of cellular reaction that were characteristic for different histologic types of lung carcinoma and that seem to be applicable to tumors of other tissues as well.

The degree of cellular infiltration was highest in squamous cell carcinomas and lowest or nonexistent in oat cell carcinomas (table 1). Within the various histologic categories the well differentiated carcinomas appeared to be accompanied by more reactive cells than the poorly differentiated.

The plasma cells were distinctively associated with squamous cell carcinomas and their number in the stroma of such tumors was proportionate to the degree of differentiation and the presence of keratin produced by the tumor. In a morphologic study of the stromal reaction of various malignant tumors, now in preparation, we have found that the selective association of plasma cells with squamous cell carcinoma occurs not only in lung tumors but in squamous cell carcinomas of all other organs so far examined (tongue, esophagus, cervix, endometrium, anus and skin).

In searching for a biologic meaning to these correlations, it appears that lymphocytes, and plasma cells accumulate in and around tumor tissues in response to specific antigenic stimulation. This is not a nonspecific reaction to infected or necrotic tissues, as it has been frequently assumed because 1. the cells responding to infected or necrotic tissues are invariably polymorphonuclears and not lymphocytes and plasma cells and 2. the present study

clearly shows that the carcinomas with the greatest amount of necrosis (oat cell, undifferentiated carcinoma) have the least amount of cellular infiltration (table 1). The correlation between degree of tumor differentiation and amount of cellular infiltrate further strengthens the assumption that the reactive cells are specifically attracted to the tumor site by tumor-associated antigens. Since undifferentiated carcinomas are not accompanied by cellular infiltrates, it may be assumed that such tumor cells are less antigenic. It is possible that, during the progression of tumors, as a result of clonal selection, new cancer cells emerge that are poorly differentiated and exhibit little or no antigenic expression. Such cells are therefore more likely to escape immunologic recognition and annihilation and to give rise to cancers of increased resistance and aggressivity.

Lymphocytes and plasma cells may respond to a large variety of tumor-associated antigens. In the case of squamous cell carcinomas the presence of large numbers of plasma cells seems to be attributable to the capacity of these tumors to produce keratin. We have documented earlier the association of plasma cells with keratinized squamous epithelia in several other contitions, not all neoplastic. We have also shown that sera of patients with pulmonary squamous cell carcinomas reacted specifically against cutaneous squamous epithelium in indirect immunofluorescence and with a soluble antigen extracted from such epithelium, in complement fixation tests (20,22).

In the present study just as plasma cells were associated with squamous cell carcinomas, deposition of dense acellular collagen appeared to be associated with oat cell carcinomas. There are tumors of other organs, infiltrating duct

carcinoma of the breast being a notable example, that appear to induce a fibro-collagenous reaction rather than a cellular infiltrate.. This might represent another pattern of reaction to tumor growth, characteristic for tumor cells of a particular antigenic expression.

At the present time we know little about the nature and variety of human tumor-associated antigens. However, as these antigens will become better defined in the future we may find that various patterns of stromal reactions appropriately match the categories of tumors and the types of antigens.

The large numbers of plasma cells and lymphocytes present in the tumor stroma and their apparently selective association with different tumor types suggested that tumor specific antibodies might be released by these cells and that the tumor site would be the logical place to attempt their recovery. This assumption was supported by several recent studies showing that low pH eluates of experimental (61) as well as of human (38, 62-65) tumors contain immunoglobulins and that these tumor-bound antibodies may block lymphocyte-mediated cytotoxicity in vitro (61, 63).

In the present study, when cryostat sections of lung carcinomas were stained with antihuman immunoglobulins in direct immunofluorescence tests the cellular infiltrates of plasma cells and lymphocytes, reacted positively for all three immunoglobulins IgG, IgM and IgA. The eluates recovered from solid lung carcinomas reacted positively in indirect immunofluorescence with tissue-cultured lung adeno and squamous carcinoma cells but not with cell suspensions of normal lung or tissue-cultured cells of 15 nonpulmonary tumors, with the exception of two squamous cell carcinomas of skin and larynx.

The results obtained with eluates of pleural effusions were even more encouraging. IgG fractions prepared from two lung carcinoma effusions produced strong cytoplasmic staining in indirect immunofluorescence with all four lung carcinoma tissue-cultured cells tested and with none of 7 cultures of nonpulmonary tumors and of 3 normal fetal and adult lungs. Eluates of 2 non carcinomatous pleural effusions did not react with lung cancer cells in indirect immunofluorescence.

Although so far only a limited number of cases have been assayed, the results were consistent in indicating that tumor specific antibodies were present at the tumor site in amounts significantly larger than in the general circulation. It also appeared that these immunoglobulins were largely the product of the plasma cells and lymphocytes accumulated in the tumor stroma. It is presently not clear whether antibodies produced against tumor-associated antigens are beneficial to the host in the fight to eradicate the tumor. Some studies indicate that such antibodies may in fact abrogate the in vitro cytotoxic activity of lymphocytes by blocking the receptor sites on tumor cells (61, 63, 66) thus enhancing the progression of the tumors (61, 63). However, final conclusions on this matter are not yet at hand since in vitro-in vivo comparisons were frequently inconclusive.

In our work we did not attempt to correlate directly the type or amount of stromal cellular reaction with clinical events such as the recurrence of tumors or survival of patients because it appeared that more than one factor must be operative in these complex events. However, having shown that the degree of cellular reactions correlates positively with the histologic differentiation of lung carcinomas, this study implicitly indicates that local cellular reactions

correlate with better prognoses, since patients with squamous cell carcinomas have longer survivals than patients with oat cell carcinomas, and well differentiated tumors appear to behave less malignantly than poorly differentiated tumors of the same histologic type. Whether these correlations indicate causal or merely coincidental relationships cannot be presently determined. Perhaps at a stage as late in the evolution of a tumor as its surgical removal, the stromal reaction may no longer reflect the capacity of the host to contain the growth of his tumor. Conversely, a correlation between the stromal reaction and the outcome of a malignant tumor might be established if the tumor progression were considered from the time of its inception, assuming that one knew how to determine the time of that event.

While these considerations might still be considered theoretical at the present time, the isolation of immunoglobulins from tumor tissues has important and far-reaching practical purposes. The recovery of tumor-associated immunoglobulins from antigen-antibody complexes can provide autologous immunoglobulins that do not require exhaustive absorption preparation, in amounts much greater than those obtainable from serum. Such immunoglobulins represent pure tumor-antibodies that can be used as highly specific reagents to detect the presence of tumor antigens. Our results with immunoglobulins recovered from solid tumors and pleural effusions in lung carcinoma (20, 22) and from peritoneal effusions in carcinoma of the ovary (18, 21, 23) indicate that the recovery of these immunoglobulins is feasible.

Obtaining pure, autologous, tumor-specific antibodies appears a most desirable aim for their potential use in cancer immunodiagnosis and immunotherapy.

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Legend of Figures

Fig. 1 Squamous cell carcinoma, well differentiated showing solid tumor cords surrounded (PERI), divided (INTER) and infiltrated (INTRA) by large amounts of plasma cells and lymphocytes. (Tumor = T. Stroma = S). x 100

Fig. 2 Oat cell carcinoma. Tumor cords surrounded by fibrous stroma lacking any cellular infiltrates. (Tumor = T. Stroma = S). x 100

Fig. 3 Squamous cell carcinoma, moderately differentiated. Island of tumor tissue (T) surrounded and heavily infiltrated by lymphocytes (L) that penetrate the tumor cells, occasionally . (Emperipolesis = E). x 100

Fig. 4 Squamous carcinoma cells surrounded and infiltrated by plasma cells and lymphocytes (Emperipolesis = E). Alterations of tumor cells indicate degeneration and death; degenerative changes = D). x 250

Fig. 5 Undifferentiated carcinoma (T) with extensive area of necrosis (N), entirely devoid of cellular infiltrates. x 250

Fig. 6 Squamous cell carcinoma, well differentiated, showing frequent cytophagocytosis (tumor cells engulfed by tumor cells).
Tumor cell partly (1) or entirely (2) degenerated within phagocytic vacuoles of tumor cells. x 250

Fig. 7 Well differentiated squamous cell carcinoma (T) with abundant stromal cellular reaction composed almost entirely of plasma cells (P); (emperipolesis = E). x 250

Fig. 8 Cryostat section of lung squamous cell carcinoma stained with FITC-labeled goat antihuman IgG in direct immunofluorescence, showing rich infiltrate of antibody-secreting cells. x 400

Fig. 9 Tissue culture of lung adenocarcinoma reacted with IgG eluted from a different lung adenocarcinoma (solid tumor), in indirect immunofluorescence, showing positive cytoplasmic and nucleolar staining. x 250

Fig.10 Immunoelectrophoresis plate comparing patterns of untreated pleural effusions (1 and 3) with patterns of immunoglobulins recovered by acid dissociation and salt pricipitation (2 and 4). Troughs (A) contain goat anti human serum antisera. Patterns 2 and 4 indicate that the recovered immunoglobulin fractions consist almost entirely of IgG.

Fig.11 Tissue culture of lung squamous cell carcinoma reacted with IgG fraction recovered from pleural effusion of lung adenocarcinoma in indirect immunofluorescence, showing positive cytoplasmic and nucleolar staining. x 400

Table 1. Stromal Cellular Reaction in Lung Carcinomas

TUMOR				INVASION			REACTION							
								Composition			Distribution			
Type[1]	Cases[2]	Differ.[3]	Necrosis	Tissues	Vessels	L.N.[4]	Amount	p[5]	L[6]	P + L[7]	Peri	Inter	Intra	Fibrosi
Squamous Carcinoma	16	3.5-well	1.3	2.4	0.6	6	3.4	9	3	4	3	3	10	3.7
	5	2.0-poor	1.4	2.6	0.8	6	2.8	1	3	1	2	1	2	2.2
Adeno Carcinoma	9	3.4-well	1.4	2.3	1.0	3	2.0	0	7	2	5	3	1	1.9
	5	1.7-poor	2.6	2.1	0.5	2	1.7	0	3	2	3	2	0	2.1
Alveolar Carcinoma	2	4.0-well	1.0	2.5	2.0	0	3.4	0	0	2	1	1	0	1.0
Undiffer. Carcinoma	6	1.0-un[8]	2.7	3.3	1.0	6	2.1	1	2	3	3	2	1	2.1
Oat Cell Carcinoma	5	1.0-un[8]	2.8	3.8	1.8	8	0.7	0	3	0	3	0	0	2.2
Metast.[9] Adeno Ca.	2	3.0-well	1.0	1.5	1.0	0	2.5	0	1	1	2	0	0	2.0

1 = Histologic Type
2 = Number of Cases
3 = Degree of Differentiation
4 = Lymph Nodes
5 = Plasma Cells
6 = Lymphocytes
7 = Plasma Cells + Lymphocytes
8 = Undifferentiated
9 = Metastatic Adeno Carcinoma (1 breast; 1 colon)

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Figures in columns: Differ., Necrosis, Tissues, Vessels, Amount, Fibrosis represent averages of estimates on a scale from 1 to 4.

Figures in column: L.N. represent quotient of total uninvolved lymph nodes divided by total of involved lymph nodes.

Figures in columns: Composition and Distribution represent number of cases.

Table 3 Immunoglobulins of Lung Carcinoma Effusions In Indirect Immunofluorescence Tests.

Cells Tested	Effusions			
	Lung adeno Ca 1	Lung adeno Ca 2	Renal Failure	Hodgkin's Disease
Lung squamous Ca (E_{14})	+	+	-	-
Lung adeno Ca ($HLuT_{29}$)	+	+	-	-
Lung adeno Ca ($HLuT_{30}$)	+	+	-	-
Lung adeno Ca ($SKLu_1$)	+	ND	ND	ND
Breast infiltrating Ca (HBr_5)	-	-	-	-
Breast infiltrating Ca (HBr_{28})	-	-	-	-
Breast infiltrating Ca ($SKBr_3$)	-	-	-	-
Cervix squamous Ca (Me 180)	-	ND	ND	ND
Ovarian Ca (OT_{98})	-	-	-	-
Ovarian Ca (OT_{101})	-	-	-	-
Melanoma (HMT_1)	-	-	-	-
Normal fetal lung (NHLu)	-	-	-	-
Normal fetal lung (NHLu)	-	ND	ND	ND
Normal adult lung (WI_{38})	-	ND	ND	ND

Ca = Carcinoma

ND = not done

Table 2 Immunoglobulins of Solid Lung Carcinoma Eluates In Indirect Immunofluorescence Tests.

Cells Tested	#	Lung Carcinoma Eluates El 1	El 2	El 3	El 4
Lung adeno Ca	- 2	2/2	0/2	2/2	2/2
Breast infiltrating Ca	- 2	0/2	0/2	0/2	0/2
Colon adeno Ca	- 3	0/3	0/3	0/3	0/3
Cervix squamous Ca	- 4	0/4	0/4	0/4	0/4
Bladder urothelial Ca	- 1	0/1	0/1	0/1	0/1
Kidney clear cell Ca	- 1	0/1	0/1	0/1	0/1
Endometrial adeno Ca	- 1	0/1	0/1	0/1	0/1
Vaginal adeno Ca	- 1	0/1	0/1	0/1	0/1
Skin squamous Ca	- 1	1/1	0/1	1/1	0/1
Larynx squamous Ca	- 1	1/1	0/1	1/1	0/1
Hepatoma	- 1	0/1	0/1	0/1	0/1
Melanoma	- 1	0/1	0/1	0/1	0/1
Fetal normal lung	- 1	0/1	0/1	0/1	0/1
Adult normal lung	- 1	0/1	0/1	0/1	0/1

Ca = Carcinoma

= Number of cases

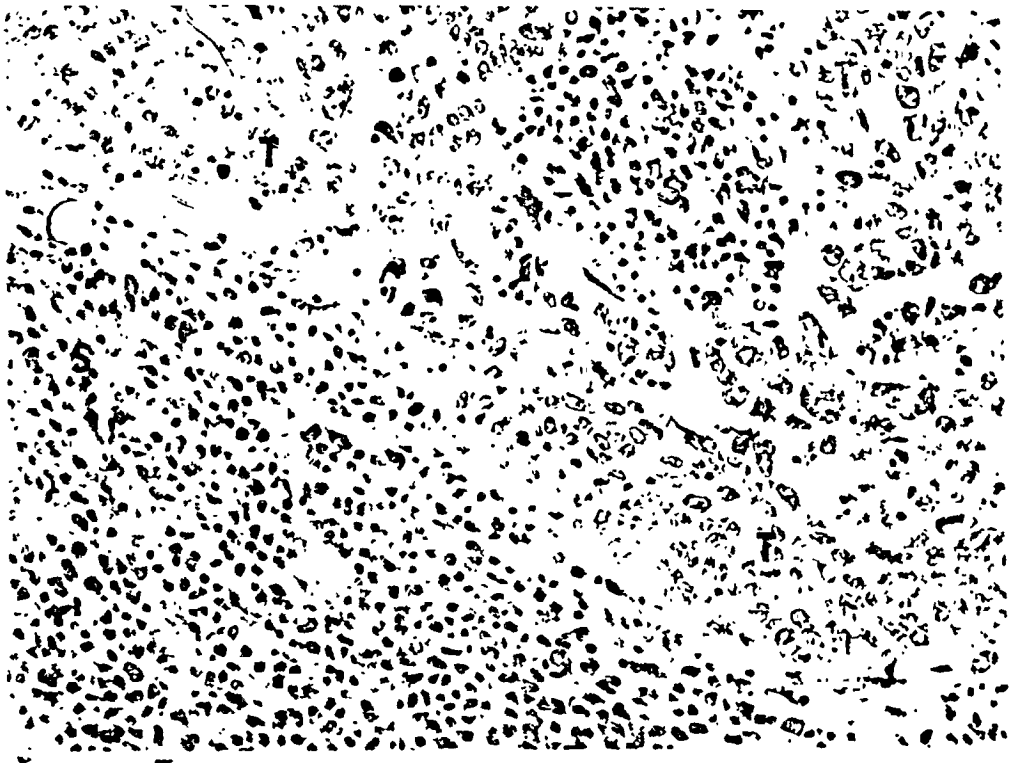

Fig 1

Fig 2

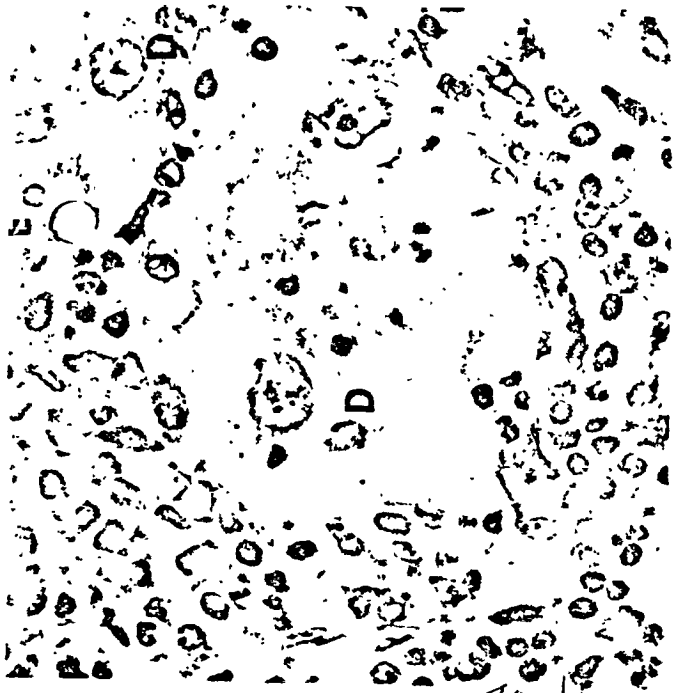

Fig 4

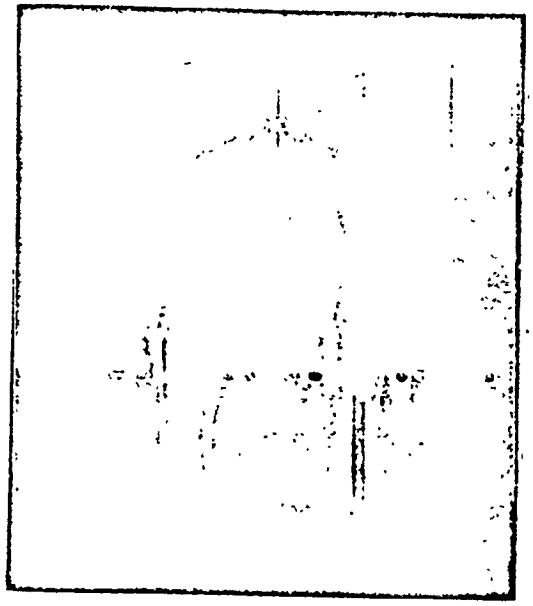

Fig 10

Fig 3

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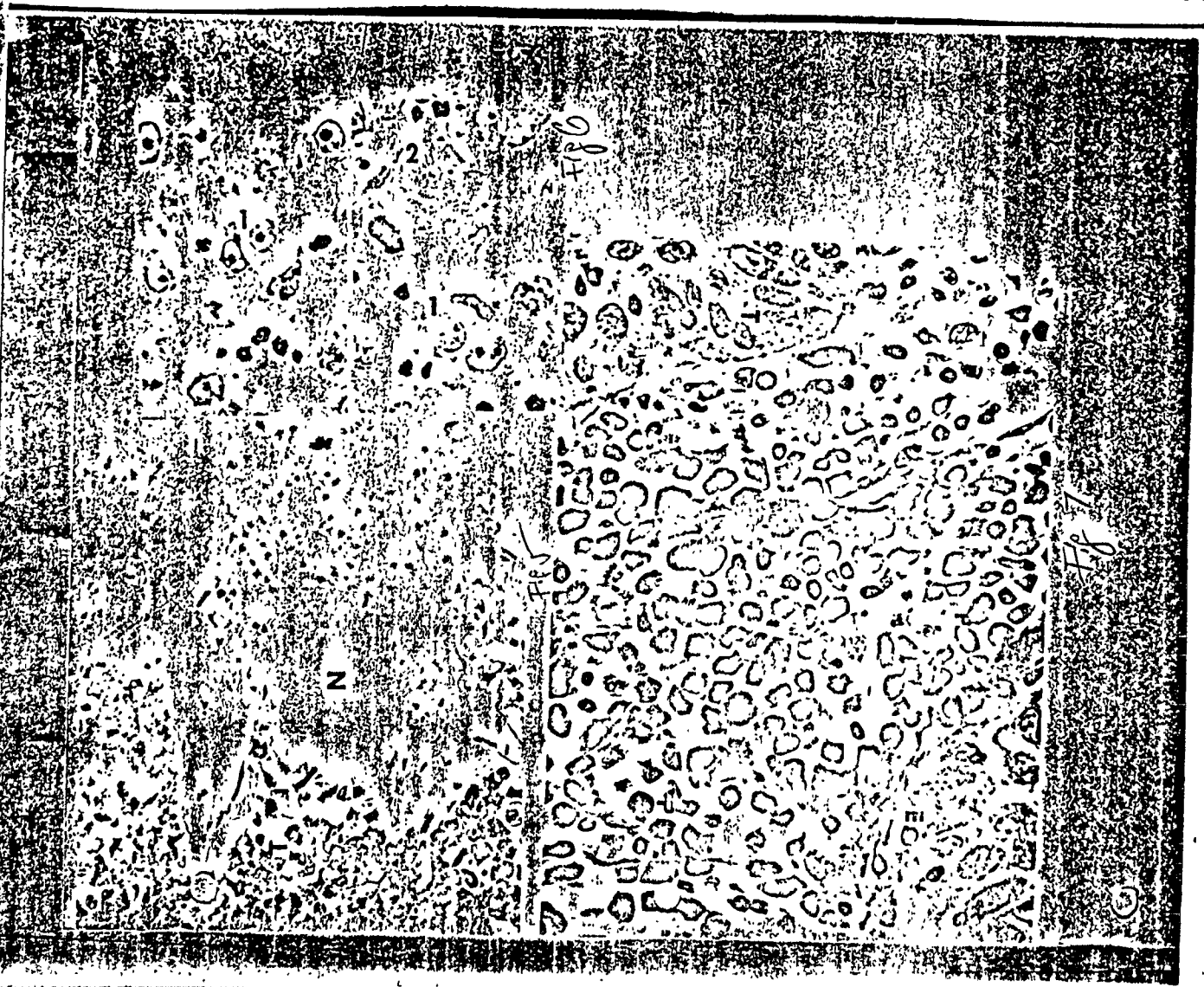

Fig 11

Fig 9

Fig 8